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SECTION A

Biotechnology for Wider Applications

CHAPTER

2

Recombinant DNA Technology

Recombinant DNA technology is recombining of DNA molecules from two different species inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, or industry. This chapter deals with the basic principles and practical applications of recombinant DNA technology that can be used to study mutations and their biological effects, gene therapy, reverse genetics, diagnostics, genomics, and protein manufacture.

Keywords: Recombinant DNA technology; genetic engineering; gene cloning; genetic transformation.

INTRODUCTION

Recombinant DNA is a form of artificial DNA that is engineered through the combination or insertion of one or more DNA strands, thereby combining DNA sequences that would not normally occur together. In terms of genetic modification, recombinant DNA is produced through the addition of relevant DNA into an existing organismal genome, such as the plasmid of bacteria, to code for or alter different traits for a specific purpose. It differs from genetic recombination in that it does not occur through processes within the cell or ribosome, but is exclusively engineered. Recombinant DNA technology utilizes techniques for cleaving DNA and splicing it into a carrier molecule (vector) that make it possible to transfer genetic information from one organism to an unrelated organism, where it may be expressed. The host cells will then synthesize the foreign protein from the recombinant DNA. When the cells are grown in vast quantities, the foreign or recombinant protein can be isolated and purified in large amounts. Recombinant DNA technology is also called Hybridization and Genetic Engineering *in vivo* and *in vitro* respectively.

The recombinant DNA technique was engineered by Stanley Norman Cohen and Herbert Boyer in 1973. They published their findings in a 1974 paper entitled "Construction of Biologically Functional Bacterial Plasmids *in vitro*", which described a technique to isolate and amplify genes or DNA segments and insert them into another cell with precision, creating a transgenic bacterium. Recombinant DNA technology was made possible by the discovery of restriction endonucleases by Werner Arber, Daniel Nathans, and Hamilton Smith, for which they received the 1978 Nobel Prize in Medicine.

Recombinant DNA technology is not only an important tool in scientific research, but it has also impacted the diagnosis and treatment of diseases and genetic disorders in many areas of medicine. It has enabled many advances, including isolation of large quantities of pure protein; identification

of mutations; diagnosis of affected and carrier states for hereditary diseases; and transferring of genes from one organism to another. Because of the importance of DNA in the replication of new structures and characteristics of living organisms, it has widespread importance in recapitulating via viral or non-viral vectors, both desirable and undesirable characteristics of a species to achieve characteristic change or to counteract effects caused by genetic or imposed disorders that have effects upon cellular or organismal processes. Through the use of recombinant DNA, genes that are identified as important can be amplified and isolated for use in other species or applications, where there may be some form of genetic illness or discrepancy, and provides a different approach to solve complex biological problem. Applying these techniques has already brought significant changes in industry and medicine. Future applications of recombinant DNA technology will transform our lives.

CONCEPTS

The history of recombinant DNA technology follows a classic pattern for scientific discoveries. At first there was a phase of slow development as a few unconnected theories were formed about fundamental biological processes. Then, quite suddenly, many of these different lines of work converged. There followed a rapid expansion as these theories led to techniques, and the techniques found valuable practical uses.

The foundation for recombinant DNA technology can be traced to Thompson's discovery of the electron in 1887 that laid the foundation for biochemistry, Miescher's isolation of DNA, Avery's demonstration of DNA as genetic material in 1944, Watson and Crick's proposal of DNA's double helix structure in 1953, plasmidology by Cohen *et al*. in 1973. The development of recombinant DNA tools and techniques in the early 1970s led to much concern about developing genetically modified organisms with unanticipated and potentially dangerous properties. This concern led to a proposal for a voluntary moratorium on recombinant DNA research in 1974, and to a meeting in 1975 at the Asilomar Conference Center in California. Participants at the Asilomar Conference agreed to a set of safety standards for recombinant DNA work, including the use of disabled bacteria that were unable to survive outside the laboratory. This conference helped to satisfy the public about the safety of recombinant DNA research, and led to a rapid expansion of the use of these powerful new technologies. Using recombinant DNA technology, first fungal gene was cloned and expressed in *E. coli* in 1976, followed by a hormone, Somatostatin, responsible for the growth, Dihydrofolate reductase and Proinsulin in 1978 and Hepatitis B genes in 1979. The list went on and continues to expand even today. Genetic engineering has now become integral part of our lives. Scientific views on DNA and genes are challenging the fundamental concepts of the nature of the life, society and humanities. The ethical and social aspects of genetic engineering are being debated intensely today. The concept of using recombinant molecules is mainly for two purposes: first, for the basic study of gene structure (e.g., discovery of the split gene in eukaryotes), and secondly to endow (or assurance) the host with the capability of producing substances, which it normally is unable to do (e.g., biosynthesis of insulin, growth hormone, interferon, etc.).

PRINCIPLES

The basic principles of recombinant DNA technology involve digesting a vehicle e.g., plasmids or viral DNA with a restriction enzyme, which is the molecular scissors that cuts DNA at specific sites. A DNA molecule from the organism of interest is also digested in a separate tube with the same restriction enzyme. The two DNAs are then mixed together and joined, this time using an enzyme called DNA ligase, to make an intact double-stranded DNA molecule. So the vehicle containing the foreign DNA is then introduced into the recipient organism by transformation or transfection. It is important to make sure that the vehicle has a replication origin, which is recognized by the DNA synthesis machinery of the host. After this the foreign DNA is multiplied manifold in the new host (Figure 2.1). It is also essential to ensure that the signals involved in the

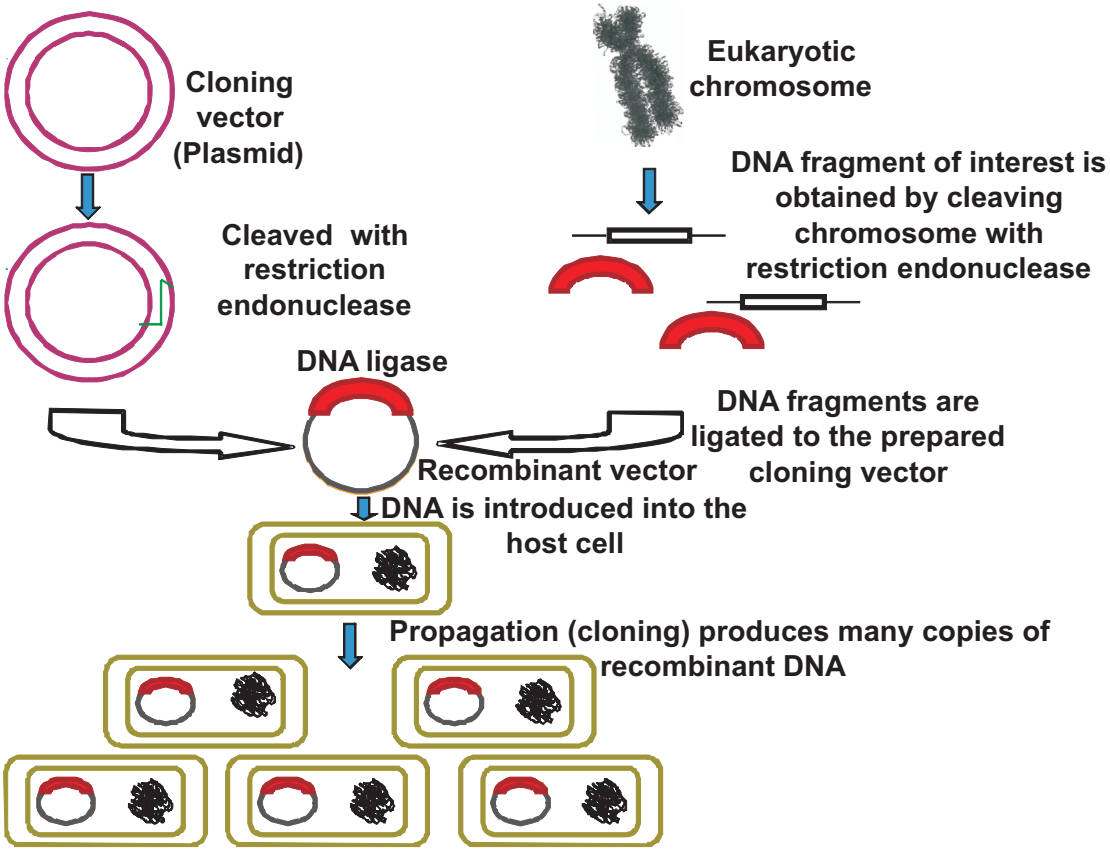


Figure 2.1 Schematic illustration of DNA cloning.

expression (transcription and translation) of the new gene are compatible with the expression machinery of the host. This novel DNA molecule is then isolated from the host cells and analyzed to make sure that the correct construct was produced. This DNA can then be sequenced, used to generate protein from *E. coli* or another host, or for many other purposes. There are many variations of this basic method of producing recombinant DNA molecules. For example, sometimes researchers are interested in isolating a whole collection of DNAs from an organism. In this case, they digest the whole genome with restriction enzyme, join many DNA fragments into many different vector molecules, and then transform those molecules into *E. coli*. The different *E. coli* cells that contain different DNA molecules are then pooled, resulting in a "library" of *E. coli* cells that contain, collectively, all of the genes present in the original organism. Another variation is to make a library of all expressed genes (genes that are used to make proteins) from an organism or tissue. In this case, RNA is isolated. The isolated RNA is converted to DNA using the enzyme called reverse transcriptase. The resulting DNA copy, commonly abbreviated as cDNA, is then joined to vector molecules and put into *E. coli*. This collection of recombinant cDNAs (a cDNA library) allows researchers to study the expressed genes in an organism, independent of non-expressed DNA.

There are five essential elements in recombinant DNA technology:

1. Specific cleavage and joining of DNA molecules derived from different sources.
2. Ligation of recombinant DNA molecule to a self-replicative gene vehicle (vector).
3. Transformation of the composite DNA molecule into a host cell and selection.
4. Confirmation of the cloned gene by physical mapping and DNA sequencing.
5. Expression of foreign gene in the host.

BASIC STEPS IN RECOMBINANT DNA TECHNOLOGY

The synthetic form of DNA is made by genetic engineering by transplanting genes from one species into the cells of a host organism of a different species. Such DNA becomes part of the host's genetic make-up and is replicated. The recombinant DNA contains the genetic code for making a particular protein of the donor organism. In the following section, essential elements of recombinant DNA technology are discussed.

Methods of Cutting and Joining DNA Molecules Derived from Different Sources

Particularly important to recombinant DNA technology is a set of enzymes (Table 2.1) made available through decades of research on nucleic acid metabolism. Two classes of enzymes lie at the heart of the general approach to generate and propagate a recombinant DNA molecule. First, restriction endonucleases recognize and cleave DNA at specific DNA sequences (recognition sequences or restriction sites) to generate a set of smaller fragments. Second, the DNA fragment to be cloned can be joined to a suitable cloning vector by using DNA ligases to link the DNA molecules together.

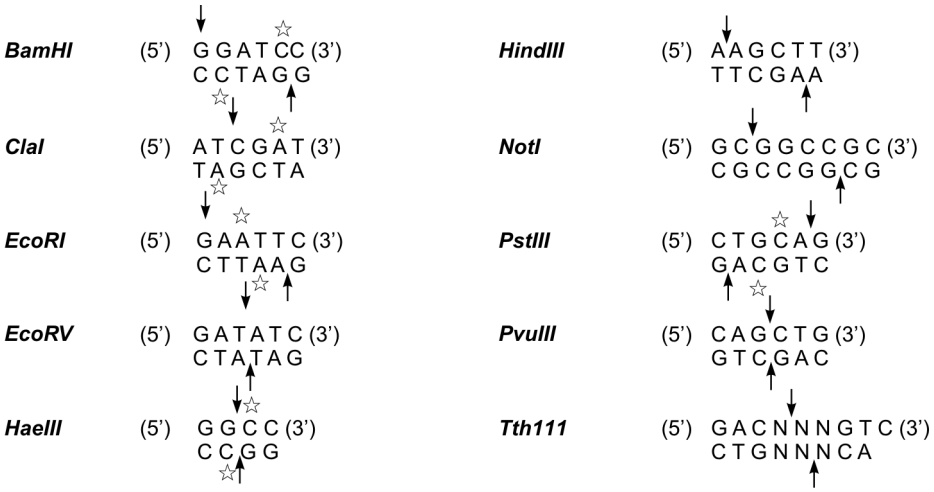
Table 2.1 Some enzymes used in recombinant DNA technology.

Enzyme	*Function*
Restriction endonucleases (type II)	Cleave DNAs at specific base sequence
DNA Ligase	Joins the two DNA molecules
DNA Polymerase I	Additions of nucleotides at 3′ ends of duplex DNA molecule for filling gaps
Reverse transcriptase	Makes a DNA copy of an RNA molecule
Polynucleotide kinase	Adds a phosphate to 5′-OH end of a polynucleotide
Terminal transferase	Adds homopolymer tails to the 3′-OH ends of a linear duplex
Exonuclease III	Removes nucleotide from the 3′ ends of a DNA molecule
Bacteriophage I exonuclease	Removes nucleotide from the 5′ ends of a duplex to expose single-stranded 3′ ends
Alkaline phosphatase	Removes terminal phosphate from either the 5′ or 3′ end (or both)

The recombinant vector is then introduced into a host cell, which amplifies the fragment in the course of many generations of cell division. Restriction endonucleases are found in a wide range of bacterial species. Werner Arber discovered in the early 1960s that their biological function is to recognize and cleave foreign DNA (the DNA of an infecting virus). Such a DNA is said to be restricted. In the host cell's DNA, the sequence that would be recognized by its own restriction endonuclease is protected from digestion by methylation of the DNA, catalyzed by a specific DNA methylase. The restriction endonuclease and the corresponding methylase are sometimes referred to as a restriction-modification system. There are three types of restriction endonucleases, designated I, II, and III. Types I and III are generally large, multisubunit complexes containing both the endonuclease and methylase activities. Type I restriction endonucleases cleave DNA at random sites that can be more than 1,000 base pairs (bp) from the recognition sequence. Type III restriction endonucleases cleave the DNA about 25 bp from the recognition sequence. Both types move along the DNA in a reaction that requires the energy of ATP. Type II restriction endonucleases, first isolated by Smith and Wilox in 1970, are simpler, require no ATP, and cleave the DNA within the recognition sequence itself. The extraordinary utility of this group of restriction endonucleases was demonstrated by Daniel Nathans, who first used them to develop novel methods for mapping and analyzing genes and genomes. Thousands of restriction endonucleases have been discovered in different bacterial species, and more than 100 different DNA sequences are recognized by one or more of these enzymes. The recognition sequences are usually 4 to 6 bp long and palindromic. Table 2.2 lists sequences recognized by a few type II restriction endonucleases. Some restriction endonucleases make staggered cuts on the two DNA strands, leaving two to four nucleotides of one strand unpaired at each resulting end. These unpaired strands are referred to as sticky ends, because they can base-pair with each other or with complementary sticky ends of other DNA fragments. Other restriction endonucleases cleave both strands of DNA at the opposing phosphodiester bonds, leaving no unpaired bases on the ends, often called blunt ends (Figure 2.2).

The average size of the DNA fragments produced by cleaving genomic DNA with restriction endonucleases depends on the frequency with which a particular restriction site occurs in the DNA molecule. In a DNA molecule with a random sequence in which all four nucleotides were equally abundant, a 6 bp sequence recognized by a restriction endonuclease such as BamHI would occur on an average once every 4^6 (4,096) bp, assuming the DNA had a 50% G-C content. Enzymes that

Table 2.2 Recognition sequences for some type II restriction endonucleases.



Arrows indicate the phosphodiester bonds cleaved by each restriction endonuclease. Asterisks indicate bases that are methylated by the corresponding methylase (where known). N denotes any base. Note that the name of each enzyme consists of a three-letter abbreviation (in italics) of the bacterial species from which it is derived, sometimes followed by a strain designation and Roman numerals to distinguish different restriction endonucleases isolated from the same bacterial species. Thus BamHI is the first (I) restriction endonuclease characterized from *Bacillus amyloliquefaciens*, strain H.

recognize a 4 bp sequence would produce smaller DNA fragments from a random-sequence DNA molecule; a recognition sequence of this size would be expected to occur about once every 4^4 (256) bp. In natural DNA molecules, particular recognition sequences tend to occur less frequently than this because nucleotide sequences in DNA are not random and the four nucleotides are not equally abundant. In laboratory experiments, the average size of the fragments produced by restriction endonucleases cleavage of a large DNA can be increased by simply terminating the reaction before completion; the result is called a partial digest. Fragment size can also be increased by using a special class of endonucleases called homing endonucleases. These recognize and cleave much longer DNA sequences (14 to 20 bp). Once a DNA molecule has been cleaved into fragments, a particular fragment of known size can be enriched by agarose or acrylamide gel electrophoresis or by HPLC. After the target DNA fragment is isolated, DNA ligase can be used to join it to a similarly digested cloning vector, i.e., a vector digested by the same restriction endonuclease. A fragment generated by *EcoRI*, for example, generally will not link to a fragment generated by *BamHI*. DNA ligase catalyzes the formation of new phosphodiester bonds in a reaction that uses ATP or a similar cofactor. The base-pairing of complementary sticky ends greatly facilitates the ligation reaction. Blunt ends can also be ligated, albeit less efficiently. Researchers can create new DNA sequences by inserting synthetic DNA fragments (called linkers) between the ends that are being ligated. Inserted DNA fragments with multiple recognition sequences for restriction endonucleases (often useful later as points for inserting additional DNA by cleavage and ligation) are called polylinkers. The effectiveness of sticky ends in selectively joining two DNA fragments was apparent in the earliest recombinant DNA experiments. Before restriction endonucleases were widely available, some workers found they could generate sticky ends by the combined action of

the bacteriophage l, exonuclease and terminal transferase. The fragments to be joined were given complementary homopolymeric tails. Lobban and Kaiser used this method in 1973 in the first experiments to join naturally occurring DNA fragments. Similar methods were used soon after in the laboratory of Paul Berg to join DNA segments from simian virus 40 (SV40) to DNA derived from bacteriophage l, thereby creating the first recombinant DNA molecule with DNA segments from different species.

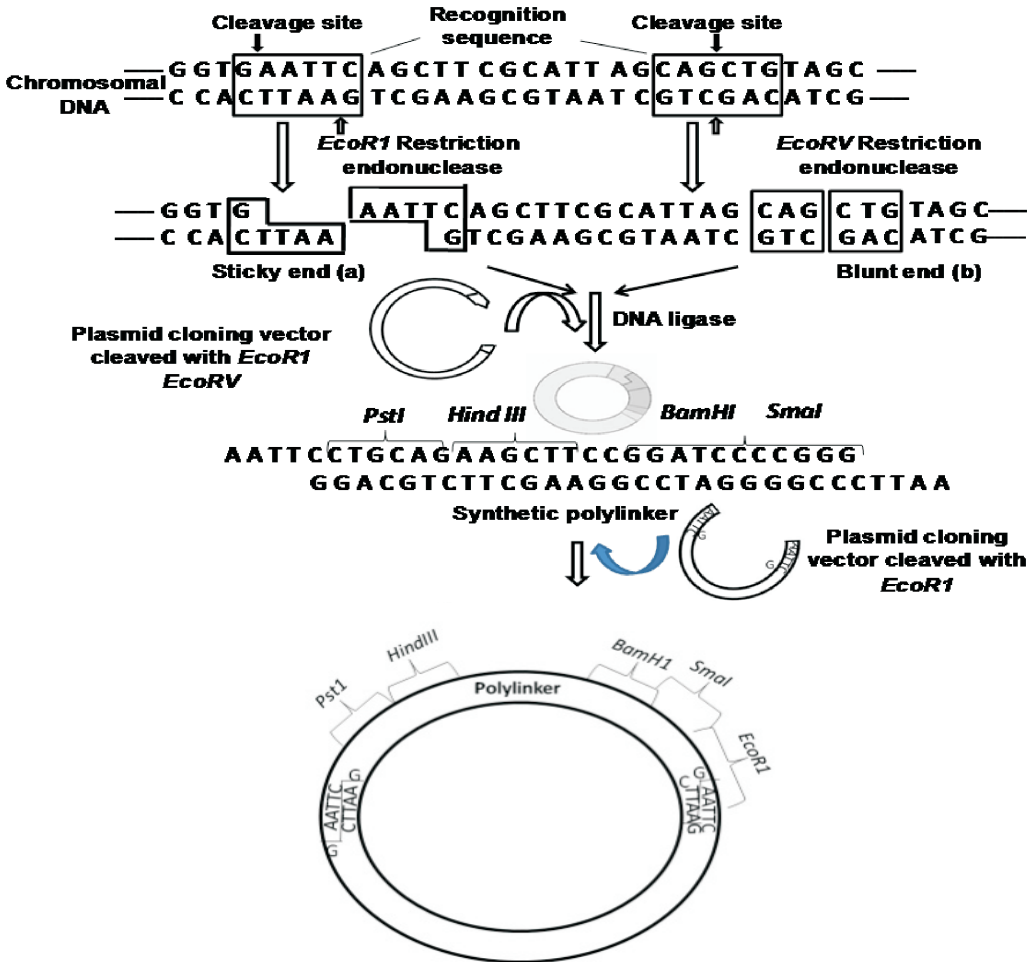


Figure 2.2 A synthetic DNA fragment (polylinker) with recognition sequences for several restriction endonucleases can be inserted into a plasmid that has been cleaved by a restriction endonuclease.

Cloning Vectors Allow Amplification of Inserted DNA Segments

Most segments of DNA do not have an inherent capacity for self-replication, and in order to reproduce themselves in a biological system, they need to integrate into DNA molecules that can replicate in the particular system (gene cloning) and for maintaining through generations. Even a

DNA segment that can replicate in its original host was not likely to have specific genetic sequences (signals) required to replicate in different hosts, so if a foreign DNA has to be propagated in bacteria, a suitable vehicle or carrier is required. They are also called vectors or cloning vehicles or carrier molecule, e.g., plasmids, cosmids, bacteriophages, M-13 vectors, shuttle vector, etc. Hundreds of different cloning vectors have been described and constructed for special purposes. However, cloning vehicles should have the following features:

- It must be able to replicate, i.e., possession of an origin of replication, which ensures that they are propagated in the desired host cell. Some bifunctional vectors used as a cloning vector into unrelated host cells, i.e., *E. coli* and *B. subtilis*, have more than one origin of replication. Some cloning vectors used in yeast carry a centromere to facilitate segregation of cell division.
- The DNA of cloning vector ideally must have only a single target site for any particular restriction endonuclease. Now commercially available cloning vectors possess multiple cloning sites which can be cleaved by several restriction endonucleases.
- There must be some way to introduce vector DNA into a cell. Generally, vectors can be introduced into the host cell by transformation or transfection.
- There must be some means of detecting its presence preferably by a plating test in Petri dishes. All useful cloning vectors have one or more readily selectable genetic markers such as antibiotic resistance to cells.

Plasmids: Extra chromosomal circular DNA molecules occur naturally in bacteria (Figure 2.3a) that replicate separately from the host chromosome and are also responsible for antibiotic resistance in bacteria (Figure 2.3b), e.g., pSC 101 (tetR), col E1 (immunity E1), pBR 322 (composite plasmid tet^R - amp^R), pUC 8/9 (amp< - lacZ), pBluescript KS+ (amp), pUC18 (amp), pBR322 (amp and tet) etc. Naturally occurring bacterial plasmids range in size from 5,000 to 400,000 bp. Investigators have developed many different plasmid vectors suitable for cloning by modifying naturally

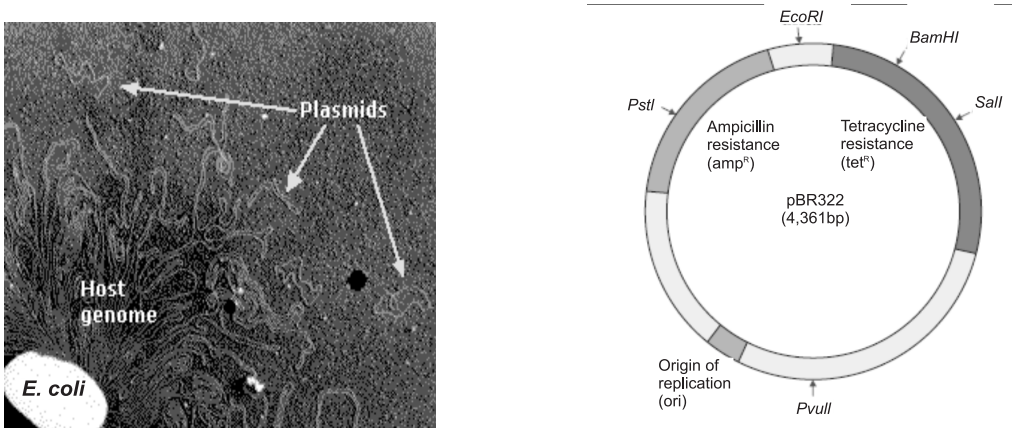


Figure 2.3 (a) Electron micrograph of an *E. coli* cell ruptured to release its DNA. The small circlets are plasmids. (Courtesy: Huntington Potter and David Dressler, Harvard Medical School). (b) The constructed *E. coli* plasmid pBR322. This was one of the early plasmids designed expressing for cloning in *E. coli.*

occurring plasmids. Transformation of typical bacterial cells with purified DNA (never a very efficient process) becomes less successful as plasmid size increases, and it is difficult to clone DNA segments longer than about 15,000 bp when plasmids are used as the vector.

Viral vectors: Bacterial viruses were though of as the most likely vehicles for genetic manipulation, however, it was the plasmid that first served as a vehicle for introducing foreign genes into a bacterium and that provided a mechanism for replication and selection of foreign DNA. Both viruses and plasmids are suitable vehicles and have been successfully used. SV40 has been the most commonly used vector for introducing foreign genes into animal cells. Virus infects animal's cells and has a covalently closed circular DNA of about 5200 bp that can be divided into "early" and "late" regions. Between the early and late regions there is a DNA sequence containing the origin of viral DNA replication. The early region is expressed throughout the lytic cycle, whereas expression of the late genes occurs only after viral DNA replication has begun.

Phage vectors: Bacteriophage λ has a very efficient mechanism for delivering its 48,502 bp of DNA into a bacterium, and it can be used as a vector to clone somewhat larger DNA segments. Two key features contribute to its utility: firstly, about one-third of the λ genome is nonessential and can be replaced with foreign DNA, and secondly DNA is packaged into infectious phage particles only if it is between 40 to 53 kb long, a constraint that can be used to ensure packaging of recombinant DNA only. Researchers have developed bacteriophage λ vectors that can be readily cleaved into three pieces, two of which contain essential genes but which together are only about 30 kb long. The third piece, "filler" DNA, is discarded when the vector is to be used for cloning, and additional DNA is inserted between the two essential segments to generate ligated DNA molecules long enough to produce viable phage particles. In effect, the packaging mechanism selects for recombinant viral DNAs. Bacteriophage λ vectors permit the cloning of DNA fragments of up to 23 kb (Table 2.3). Once the bacteriophage λ fragments are ligated to foreign DNA fragments of suitable size, the resulting recombinant DNAs can be packaged into phage particles by adding them to crude bacterial cell extracts that contain all the proteins needed to assemble a complete phage. This is called *in vitro* packaging. All viable phage particles will contain a foreign DNA fragment. The subsequent transmission of the recombinant DNA into *E. coli* cells is highly efficient.

Cosmids: (Phage cos-site carrying plasmids) These are novel vectors that combine the features of a plasmid and phage to increase the probability of selecting a recombinant plasmid carrying foreign DNA. A typical cosmid is circular col E1 plasmid carrying both the gene for resistance to the drug rifampicin (rif^R) and the cos-site of phage. The cosmid has two cleavage sites for the *Hind III* restriction enzyme, which separate the *rif* gene from the cos site and the col E1 region. Cloning the eukaryotic genome in phage limits the size of the eukaryotic segment that can be cloned to about 15 kilobases (kb), i.e., smaller genes. However, for cloning larger gene of 35 to 40 kb, phage is not suitable. Therefore, cosmids are generally used for this purpose (Table 2.3). A technique for cloning much larger eukaryotic fragments in *E. coli* is known as "cosmid cloning". The cos sites are necessary for packaging DNA into phage heads. The cos sites of phage have been cloned into ampicillin resistance gene of pBR322, leaving the tetracycline gene intact.

Bacterial artificial chromosomes (BACs): Bacterial artificial chromosomes are simply plasmids designed for the cloning of very long segments (typically 100,000 to 300,000 bp) of DNA (Table

2.3). They generally include selectable markers such as those having resistance to the antibiotic chloramphenicol (Cm^R), as well as a very stable origin of replication (ori) that maintains the plasmid at one or two copies per cell. DNA fragments of several hundred thousand base pairs are cloned into the BAC vector. The large circular DNAs are then introduced into the host bacteria by electroporation. These procedures use host bacteria with mutations that compromise the structure of their cell wall, permitting the uptake of the large DNA molecules.

Yeast artificial chromosomes (YACs): *E. coli* cells are by no means the only hosts for genetic engineering. Yeasts are particularly convenient eukaryotic organisms for this work. The genome of the most commonly used yeast, *Saccharomyces cerevisiae*, contains only 14×10^6 bp (less than four times the size of the *E. coli* chromosome), and its entire sequence is known. Yeast is also very easy to maintain and grow on a large scale in the laboratory. Plasmid vectors have been constructed for yeast, employing the same principles that govern the use of *E. coli* vectors described above. Convenient methods are now available for moving DNA into and out of yeast cells, facilitating the study of many aspects of eukaryotic cell biochemistry. Research work with large genomes and the associated need for high-capacity cloning vectors led to the development of yeast artificial chromosomes (YAC). YAC vectors contain all the elements needed to maintain a eukaryotic chromosome in the yeast nucleus: a yeast origin of replication, two selectable markers, and specialized sequences needed for stability and proper segregation of the chromosomes at cell division. Before being used in cloning, the vector is propagated as a circular bacterial plasmid. Cleavage with a restriction endonuclease *(BamHI)* removes a length of DNA between two telomere sequences (TEL), leaving the telomeres at the ends of the linearized DNA. Cleavage at another internal site *(EcoRI)* divides the vector into two DNA segments, referred to as vector arms, each with a different selectable marker.

Table 2.3 Cloning capacity of commonly used vectors.

Vector	*Insert size range (Kb)*
Plasmid	≤ 10
λ Phage	≥ 23
Cosmid	30-45
P1 artificial chromosome (PAC)	130-150
Bacterial artificial chromosome (BAC)	≤ 300
Yeast artificial chromosome (YAC)	200-2000

Shuttle vectors: A shuttle vector is a vector that can replicate in different organisms. The first shuttle vector, which contained *E. coli* and yeast components, was used to clone yeast genes. If a yeast gene were cloned into an *E. coli* plasmid and then *E. coli* cells were transformed by the recombinant plasmid, in general the yeast gene would not be expressed for usual reasons–lack of recognition of yeast promoters in *E. coli* and incorrect processing. Shuttle vectors, which contain sequences from *E. coli* plasmid and a particular region of yeast genome, were designed to avoid this problem. Essential features of this shuttle vector are two replication origins (one active in yeast and one in *E. coli*), two selective markers (trp, detectable in yeast, and amp, detectable in *E. coli*), and restriction sites next to a yeast promoter. A problem with these vectors is that they are not particularly stable in yeast because they lack a centromere (the portion of chromosome by which the chromosome is attached to the mitotic spindle), thus replicas are not efficiently segregated into daughter cells.

Expression vectors: An expression vector, otherwise known as an expression construct, is generally a plasmid that is used to introduce and express a specific gene into a target cell. Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cellular transcription and translation machinery. The plasmid is engineered such that it contains a highly active promoter, which causes the production of large amounts of mRNA. Some important expression vectors used now-a-days are pBI121, pCAMBIA, pgD–DHFR, etc.

Different Cloning Strategies

DNA technique for placing and maintaining a new gene in bacteria is called cloning i.e., isolation of a specific new DNA sequence from one organism and placing this gene into another organism with the help of a vehicle that proliferates to form a population of identical descendants clone. Three approaches were generally used for cloning—shotgun, cDNA cloning and synthetic gene cloning. A comparison has been made between these cloning strategies in Table 2.4.

Shotgun: Theoretically it is possible to clone any desired gene in one organism from another organism by the technique known as shotgunning. In this case, we take the entire genome of the first organism and digest it with a restriction endonuclease to produce a random mixture of fragments. The vehicle is also cleaved off by the same restriction endonucleases, and hence we get sticky ends of foreign DNA and vehicle that can be ligated and the recombinant plasmids transformed into the desired host cell. Since each recombinant plasmid will contain a different fragment of foreign DNA, it is a major task to select those transformed cells that carry the cloned gene of interest.

cDNA cloning: The next approach is that the desired gene is present somewhere on the DNA of the eukaryotic cell. The eukaryotic DNA is divided into exons and introns and the information is broken. In prokaryotes, genes have a very simple structure and all the genetic information between the initiation and stop codon is translated into protein. In eukaryotes, genes containing exons are transcribed into mRNA in the usual manner, but the corresponding intron sequences are spliced out. As bacteria cannot splice out introns, they cannot be used directly to express many genes from mammals or other eukaryotes. One solution to the problem of introns is to clone the gene of interest in yeast, which can mediate splicing. Unfortunately, cloning directly into yeast is much less efficient than cloning into bacterium such as *E. coli* and this technique is not favored. A better approach is to start by isolating mRNA than the DNA from the original organism, e.g., isolation of mRNA from the human pancreatic cells in case of insulin, as these are rich in insulin specific m-RNA from which introns have already been spliced out. So if we have a mixture of mRNA, prepare cDNA from it and then integrate into cloning vector. Isolate the positive clones on a medium having a particular drug for which the cloning vector is carrying resistance. From these clones, isolate DNA, denature them, and add the mixture of mRNA that was earlier used to form cDNA. Keep the temperature to hybridizing temperature and then transfer it to a medium containing all translation machinery and radioactive labeled amino acids. At last, add antibody attached to beads. If the clone contains the right gene, all the mRNA complementary to it will be involved in hybridization and hence there will not be the synthesis of that particular protein. When we add antibodies, it will not find its correct protein to bind and remains as such, whereas in all other fractions, in which the

gene of interest is absent and hence no hybridization of its mRNA and hence synthesis of that protein that will be bound by antibodies, proves that it is not a gene of our interest. Using the enzyme reverse transcriptase it is possible to convert this m-RNA into a DNA copy. This copy DNA (cDNA) that carries uninterrupted genetic information for insulin production can then be cloned. This is cDNA, has blunt ends instead of sticky ends but it still can be inserted into plasmid vector using blunt ends ligation.

Synthetic gene cloning: An alternative way of avoiding the problem of introns is to synthesize an artificial gene in the test tube starting with deoxy ribonucleotides. If we know the amino acid composition of the protein, we can predict its mRNA and hence DNA. In practice various oligo-nucleotides are synthesized and ligated together before insertion into vehicle. But this approach is applicable only for small proteins (up to 500 amino acids long). For larger proteins it is difficult to synthesize long synthetic DNA in test tube. For making eukaryotic protein in bacterial cells, i.e., eukaryotic to prokaryotic transfer, a fragment of animal DNA that encodes the protein is inserted into a plasmid, a small, circular piece of bacterial DNA, which in turn serves as the vehicle for introducing the DNA into the bacterium.

Table 2.4 Advantages and disadvantages of different cloning strategies.

Cloning method	*Advantages*	*Disadvantages*
Shotgun method	Easy to do	(1) Many different clones are isolated, so a good selection method is needed to isolate the desired clone. (2) Genes with introns will not be correctly expressed in *E. coli,* the usual shotgun cloning host. (3) Expression dependent on recognition of foreign promoters by *E. coli.*
c-DNA cloning	(1) If desired mRNA is in abundance in the mRNA preparation used, desired clone should be easily detected. (2) Introns are not a problem as mRNA will contain spliced molecules	(1) Desired mRNA not always abundant so selection may be necessary. (2) Technically more difficult than shotgun cloning. (3) Cloned gene needs to be placed downstream from a promoter, since normal promoter eliminated by starting with mRNA. (4) Codon choice may not be optimal for *E. coli.*
Gene synthesis	(1) No selection needed after cloning (2) Sequence of promoters, ribosome binding sites etc. can be optimized. (3) Codon choice can be optimized for preferred host cell.	(1) Need to know protein sequence before synthetic gene can be designed.

A Means of Transferring Recombinant DNA into Functional Cells

Manipulating the sequence of gene in a test tube is not sufficient for discovering its functions. It is necessary to study the functioning of the gene in a living cell, and this requires persuading a cell to take up the gene. Fortunately many bacterial and yeast species can be directly transformed with DNA. The efficiency of the transformation has been improved by first treating bacteria with salts such as calcium chloride, adding plasmid DNA, and then exposing the cells to a brief heat shock, e.g., 42°C for 90 sec. Another effective approach for gene transfer is electroporation, in which cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm and then passes to the nucleus. There are a wide variety of methods used to transfer DNA directly in the cells. Perhaps the simplest to understand, although quite challenging to do, is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles. When first developed, microinjection was a time-consuming process and only a few cells could be injected in a single experiment. Once again, computers have revolutionized techniques. In this case, computer-controlled, automated injection means that hundreds of the cells can be injected. The first method used for introducing DNA into animal cells en masse was to incubate the DNA with an inert carbohydrate polymer (dextran) to which a negatively charged chemical group (DEAE [diethylaminoethyl]) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles in turn stick to the surface of the cells, which are though to take them in by a process known as endocytosis—a normal feature of membrane turnover. DNA can also be incorporated into artificial lipid vesicles called liposomes, which fuse with the cell membrane, delivering their contents directly to the cytoplasm.

A plant cell presents a special challenge to DNA transfer—they are encased in a thick cell wall made up of cellulose that poses a formidable barrier to efficient gene transfer. One approach is to remove this wall using fungal cellulase enzymes. The resulting protoplast is enclosed only by a plasma membrane and is much more amenable to experimental manipulations including transfection and electroporation. Protoplasts will take up macromolecules like DNA, and they are capable of regenerating whole plants, as are nearly all plant cells. In a stunningly direct approach, DNA can be absorbed on the surfaces of tungstun or gold microprojectiles, 1 μm in diameter, which is fired into intact cells as through pellets from a shotgun. Although first developed for plants, gene guns are also used to introduce DNA directly into animal cells in tissues. However, a number of nonlytic viruses drawn from the retrovirus, adenovirues, and adeno-associated virus groups have been modified to transfer genes to animal cells. Retroviruses after infecting cells use reverse transcriptase to convert their RNA genome to DNA. This viral DNA is efficiently integrated into the host genome as a "provirus", where it permanently resides, replicating along with the host DNA at each cell division. The most popular method for DNA transfer into many types of plants uses *Agrobacterium tumefaciens*, a bacterium that infects plant cells and drives the infected cells to multiply wildly, thus producing the plant tumors known as crown galls. The tumor-inducing agents in Agrobacterium are a plasmid, called Ti that integrates some of its DNA (T-DNA) into the chromosomes of its host plant cells. Ti plasmids are (200 kb large) double-stranded circular DNA that replicate independently in host cells. The process of transfer from the bacterial cells to the plant cells is mechanistically similar to the process of bacterial conjugation.

Methods of Selecting Recombinants Containing Right Gene

The task of isolating a desired recombinant from a population of transformed bacteria depends very much on the cloning strategy that has been adopted. For instance, if a synthetic gene has been cloned, no selection is necessary because every transformed cell will contain the correct sequence. When a cDNA derived from a purified or abundant mRNA is to be cloned, the task is relatively simple: only a small number of clones need to be screened. Isolating a particular single-copy gene sequence from a complete mammalian genomic library requires techniques in which hundreds of thousands of recombinants can be screened. A number of different methods have been devised to facilitate screening of recombinants. These include genetic, immunochemical and nucleic acid hybridization methods.

Genetic method: The simplest example of a genetic method is the complementations of nutritional defects, e.g., suppose a bacterial strain is available, which has a mutation in a gene encoding an enzyme involved in the biosynthesis of the amino acid histidine. Such a mutant strain, will grow

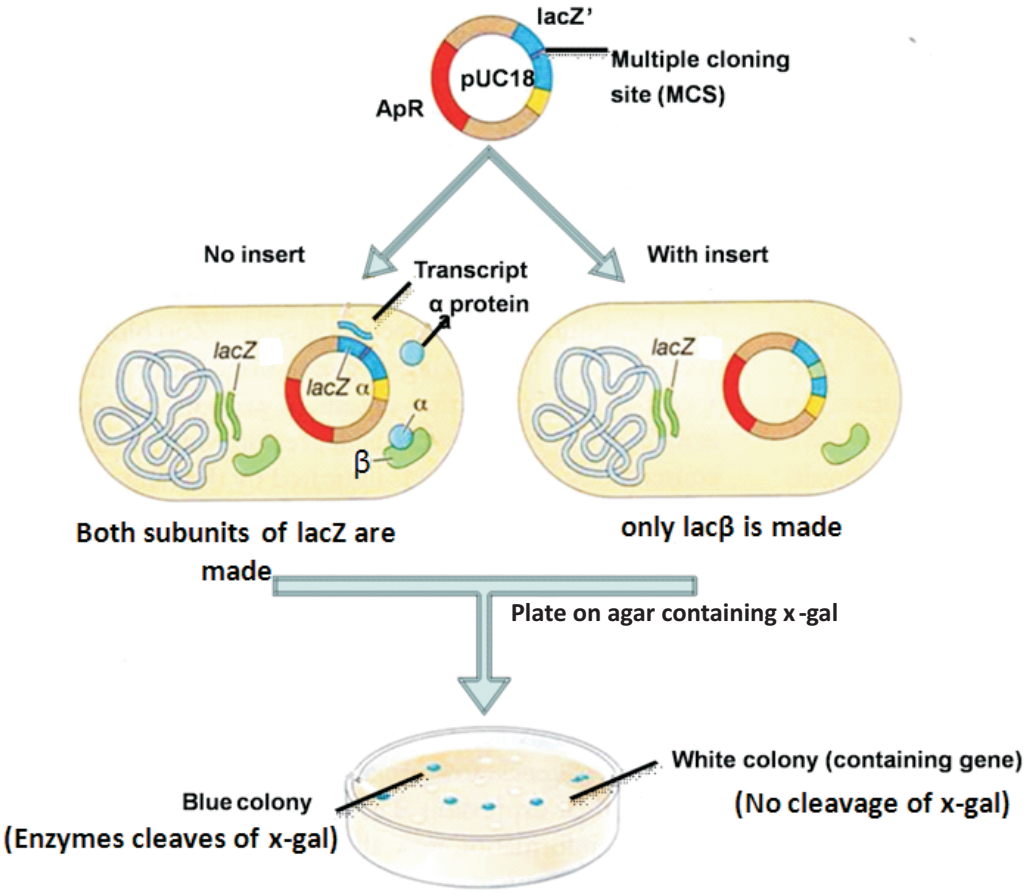


Figure 2.4 Blue/white screening to recognize vector containing inserts.

only in a medium supplemented with histidine. By cloning DNA from a normal strain, i.e., one that can synthesize its own histidine, in the mutant strain and selecting those transformants, which grow in the absence of histidine, it is possible to isolate the gene of interest. Even yeast and mammalian genes can be selected in this way. In another approach, marker genes were used in the plasmid having resistance towards antibiotic, so that the cells which have been transferred with the plasmid can be easily selected due to the death of the other unlabelled cells. Cells transformed with plasmid DNA reproduced themselves normally and produced a clone of bacteria, since each cell in the clone contained a DNA species having the same genetic and molecular properties as the plasmid DNA molecule that was taken up by a single bacterial cell. The procedure made possible the cloning of individual molecules even if they were originally present in a heterogeneous population of plasmids. In addition, blue/white screening can also be used to determine whether recombinant vector is present at high frequency in the library and picking white colonies or plaques excludes those containing empty vectors. This method relies on the β-galactosidase gene from the *lac* opern. The enzyme β-galactosidase hydrolyses the chemical X-gal to produce an insoluble blue dye. The cloning site of many vectors is within a copy of the β-galactosidase gene, so that the insertion of

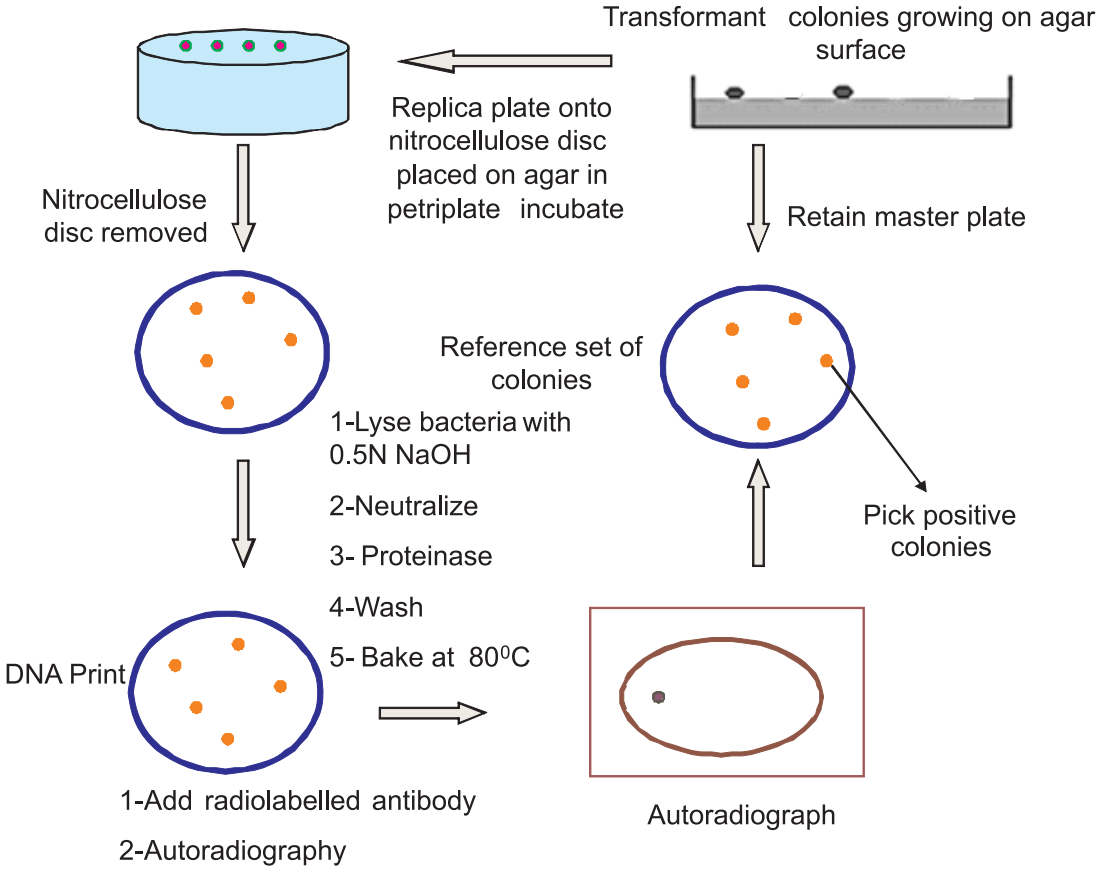


Figure 2.5 The basic method of immunological screening of recombinant.

the DNA disrupts the gene and bacterial colonies or phage plaques remain colorless (white) if X-gal is included in the culture medium. In contrast, colonies or plaques containing vectors without DNA inserts turn blue (Figure 2.4).

Immunological method: These are widely used to select particular recombinant clones, but demand the availability of a specific antibody against the desired protein product. When such antibody is available, the method works well as nucleic acid hybridization method. Transferred cells are grown on agar in a conventional Petri dish and a duplicate set of colonies prepared in a second Petri dish. Both duplicate sets of colonies are lysed by exposure to chloroform vapour and the released proteins blotted onto an absorbent matrix are then exposed to antibody, which has been radioactively labeled *in vitro* (Figure 2.5). Positively reacting lysates are detected by washing surplus radiolabelled material off the matrix and making an autoradiographic image. Many different variations of this technique have been adopted as radioactive antibody test for insulin producing colony and immunoprecipitation test for other protein producing colony.

Nucleic Acid Hybridization Method: DNA hybridization is the most common sequence-based process for detecting a particular gene or a segment of nucleic acid. There are many variations of the basic method, most making use of a labeled (such as radioactive) DNA or RNA fragment, known as a probe, complementary to the DNA being sought. However, if the protein product of a gene has been purified, probes can also be designed and synthesized by working backward from the amino acid sequence, deducing the DNA sequence that would code for it. Now, researchers typically obtain the necessary DNA sequence information from sequence databases that detail the structure of millions of genes from a wide range of organisms. Usually following three types of hybrid complexes were seen for screening particular gene of interest from whole genome.

- DNA-DNA: A single-stranded DNA molecule (ssDNA probe) can form a double-stranded, base-paired hybrid with ssDNA target if the probe sequence is the reverse complement of the target sequence. A radiolabeled DNA probe can be applied to DNA from a gel transferred to a membrane, called-Southern Blot.
- DNA-RNA: A single-stranded DNA (ssDNA) probe molecule can form a double-stranded, base-paired hybrid with an RNA (RNA is usually a single-strand) target if the probe sequence is the reverse complement of the target sequence. RNA can be radiolabeled to probe a Southern Blot or ssDNA probe can be applied to membrane-bound RNA, called-Northern Blot.
- Protein-Protein: An antibody probe molecule (antibodies are proteins) can form a complex with a target protein molecule if the antibody's antigen-binding site can bind to an epitope (small antigenic region) on the target protein. In this case, the hybrid is called an 'antigen-antibody complex' or 'complex' for short. A radiolabeled antibody can probe membrane-bound proteins, called-Western Blot.

Expression of Foreign DNA into Host

This is a big problem in genetic engineering. We can clone and identify DNA fragments carrying the information for a particular protein. Will this information work in bacteria? One of the prerequisites of genetic engineering is to use mutants, which are restrictionless so that the foreign gene inserted in bacteria cannot be cleaved by bacterial restriction endonucleases and not modified by

modification enzymes present in the bacteria. Regulatory signals, "where to start" and "where to stop" should be provided to the bacteria. For the expression of foreign DNA, all genes have two stop and two start signals—one for transcription and one for translation. A simple way to make the new protein sequences is to cut open the bacterial gene by restriction endonucleases and insert the new DNA there. So a hybrid protein will be formed. This has been done successfully in case of Somatostatin, a growth hormone. The DNA for those 14 amino acids and a stop signal was inserted. The bacterium made the hybrid protein that was cleaved off and purified to get a functional growth hormone. For getting maximum gene expression of a mammalian gene in a microorganism for facilitating commercial production of the corresponding gene product, some important factors are required:

(1) The number of copies of the plasmid vector per unit cell (copy number)
(2) The strength of the promoter
(3) The sequence of the ribosome binding site (rbs) and flanking DNA
(4) Codon choice in the cloned gene
(5) Genetic stability of the recombinant
(6) Proteolysis

The limiting factor in expression is the initiation of protein synthesis. Increasing the number of plasmids per cell increases the number of mRNA molecules transcribed from the cloned gene, and this results in increased protein synthesis. Similarly, the stronger the promoter, the more mRNA molecules are synthesized. The nucleotide base sequence of ribosome binding sites and the length and sequence of the DNA between the ribosome binding sites and the initiating AUG codon are so important that a single base change, addition or deletion can affect the level of translation up to 1000-fold. Another important factor is related to the redundancy of the genetic code. There are several trinucleotide codons for most amino acids, and different organisms favor different codons in their genes. If genes inserted into cells of another species utilized codons are in the host cell, the host's biosynthetic machinery may be starved of charged t-RNAs. This could result in premature protein chain termination or a high error frequency in the amino acid sequence of the protein. Heavy secretor of cells i.e. excess protein synthesizing cells have tendency to convert into non-secretor by spontaneous changes in gene sequence e.g. deletions and gene rearrangements. Thus it is advised to minimize recombinant gene expression by using controllable copy number. Example of controllable promoter is *E. coli* lactose operon, no transcription occurs unless lactose is added. Runaway plasmids are examples of vectors with controllable copy number. At low temperature, e.g., below 30°C, the copy number may be as low as 10 plasmids per cell, but when temperature is raised to 37°C, the copy number increases to several hundreds, or even several thousands. The enzymatic breakdown of protein (proteolysis) does not affect transcription and translation but influences the apparent rate of gene expression. Although proteolysis can be reduced, it is difficult to eliminate completely. One approach used widely is to 'protect' the desired protein by fusing it to a normal cellular protein, from which it must subsequently be released.

APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY

There are many things that can be done more effectively with the help of cloning techniques. These include gene mapping, controlled mutagenesis and production of specific gene products. However, the major revolution occurred in eukaryotic biology with the development of DNA cloning that

allows defining segments of any DNA to be isolated and characterized. Some applications of this technology in medical, veterinary, agricultural sciences and industrial applications that have possibly or greatly facilitated by DNA cloning are discussed.

Medical and Veterinary Applications

Diagnosis of genetic disease: One of the major medical applications of gene cloning is to safely predict or diagnose the genetic diseases in fetuses, which are untreatable by available therapeutic strategies, so that abortion can be offered to prevent the birth of incurably sick children. Several genetic diseases that can currently be diagnosed prenatally are various enzyumopathies that include inborn error of metabolism e.g., Tay Sachs disease, Gaucher's disease, Hurler's Syndrome, Galactosemia, hypercholesterolemia, Lesch-Nyhan Syndrome, Xerodema pigmentosum, emphysema, cystic fibrosis, muscular dystrophy, hemoglobinpathies such as sickle cell anaemia and β-thalassemias etc. The basic approach to fetal diagnosis is to obtain fetal cells—either fibroblasts or blood cells taken by amniocentesis (Figure 2.6)—and to test them for biochemical defects. Currently the tests are made for the gene products such as proteins or enzymes suspected of being defective rather than mutated gene themselves. It is also possible now to detect lethal recessive mutations directly on genome itself.

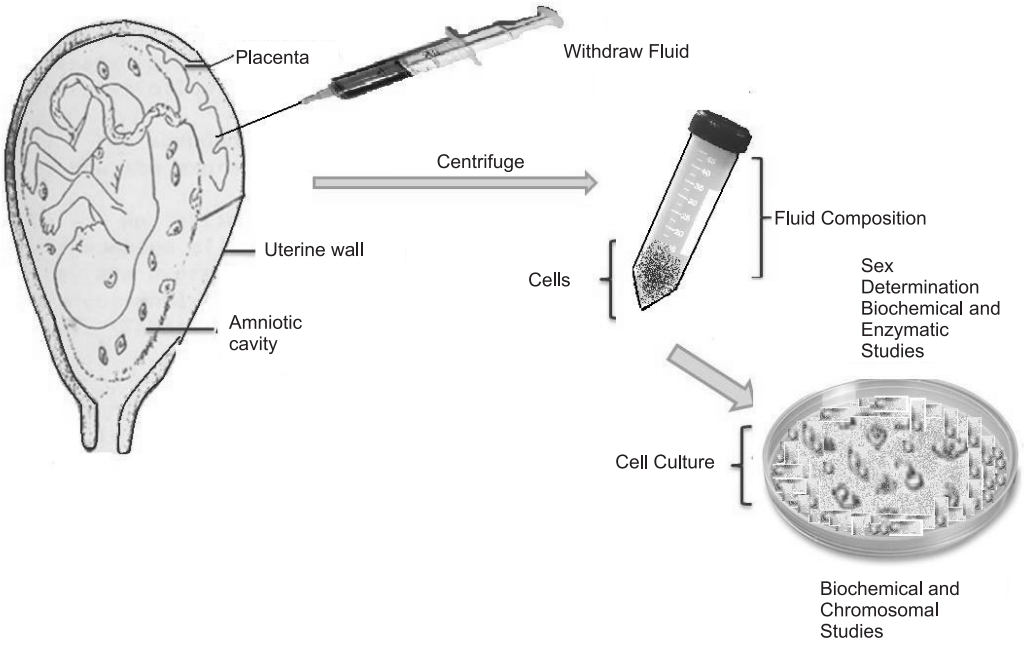


Figure 2.6 Amniocentesis. A sample of amniotic fluid is taken by inserting a needle into the amniotic cavity during or around the 16th week of gestation for diagnosis of the genetic diseases.

Gene therapy: It is the correction of an inborn error of metabolism by the insertion of a normal gene of either somatic or germ cells into the afflicted organism. In somatic therapy, healthy copy of a gene is inserted into somatic cells that are not involved in reproduction, and such an alteration is not carried to future generation. In contrast, germ-line therapy treats cells that make eggs and sperm, therefore affecting all future offspring. In gene therapy, cells need to be extracted from the body, grown in culture, genetically manipulated and then re-implanted into the patient from whom original tissue was taken. The first human experiment was carried out at NIH in 1990. The patient was born with virtually no natural protection against disease because her immune system lacked a critical gene. After inserting critical gene by gene therapy, the patient's body began generating a normal immune response. A second human experiment used altered genes to attack advanced melanoma, a disease that is always fatal. In this case, doctors removed from patients a type of cell called tumor infiltrating lymphocytes or TIL, which the body uses to attack cancers. A gene that manufactures a potent cancer fighter called tumor necrosis factor was inserted into the TIL cells. These cells were reproduced in millions and infected into the patients. Researchers hope that TIL cells will migrate naturally to the tumors and cure cancer. In 1993, Carly Todd had a single gene disease called adenosine deaminase (ADA) deficiency, in which her body could not produce working copies of the enzyme ADA because of a fault in the gene sequence. This defect has been cured successfully by implanting in her a genetically altered ADA gene containing bone marrow cells. Researchers at the National Institutes of Health reported that their work with laboratory rats showed that the flawed gene that causes cystic fibrosis can possibly be corrected in the lungs. The problem is that lung cells cannot be removed from the body, genetically altered and then returned. Somehow, the correct genes have to be inserted into the lung cell while they remain in place. The NIH team showed that this can be done in mice by placing the correct gene into the genetic make-up of cloned virus, which is then sprayed into the lungs. The virus is altered so that it does not cause infection, but is able to attack the cells in the lung. When this happens, the virus deposits the new gene into the DNA of the lung cells. With the new gene, the lung cells secrete a protein that prevents the deadly mucous from building up. In addition, Ohio University researchers have shown that gene can be inserted into fertilized mouse eggs, which will cause the rodents to be born with a genetic protection against a leukaemia virus by secreting a protein that keeps it from reproducing. Researchers caution that it will take decades before gene therapy can be used against most of the 4,000 genetic-related disorders. From birth defects in newborns to cancer in the elderly, several diseases can be cured by gene therapy, e.g., hemophilia, Lesch-Nyhan disease, Immuno-deficiency and combined immunodeficiency disease which are due to deficiency of factor VIII, Hypoxanthine-guanine phosphoribosy1 transferase (HGPRT), Pyramiding nucleoside phosphory-lase and adenosine deaminase.

Production of transgenic organisms: Recombinant DNA technology has made it possible to create transgenic organisms to produce human proteins, e.g., insulin, hemoglobin, etc. The aim of transgenesis is to produce organism with a hereditable change in their genotype such that the benefits of gene manipulation can be passed to their offspring. With mammals, the technique used is the microinjection of DNA into fertilized eggs followed by implantation of the manipulated ova in a foster mother. Scientists have introduced hemoglobin genes into pigs and made them produce human hemoglobin. In this method, the sows were first hormonally treated to produce a large numbers of eggs, which were then cultured *in vitro* with vectors carrying hemoglobin genes were

then implanted into other sows specially prepared by hormones to receive their eggs. The blood of offspring of transgenic pigs had 15-20% of human Hb. Similarly, genetic engineers have successfully introduced the two human hemoglobin genes into Brewers yeast "*Saccharomyces cerevisiae*" and have got them to produce large amounts of human hemoglobin. *E. coli* is also presently being made to produce separately the two peptide chains of hemoglobin, which are then linked up with the iron containing heme to make up the complete hemoglobin. This technique has been used to place gene that codes for alpha-1 antitrypsin into a sheep embryo. As a result, this transgenic sheep produces milk that contains the protein that is used to treat patients with a particular type of inherited lung disease, congenital emphysema. In addition, it has also been used to manufacture bovine somatotrophin (BST), a hormone that increases by 10 to 15 % the amount of milk that cow can produce.

Agricultural Applications

Recombinant DNA technology is being applied to increase yields of plants, resistance to disease or pollution and to create new crops that can utilize previously wasted resources. New gene can be inserted to a section of a bacterium's DNA to transfer the genes to the plant cells.

Improving yields: There are different ways of increasing yields. You could either aim for a faster rate of growth, more efficient use of nutrients, or an increase in the quantity of the food produced by plants. In the case of cereal crops like wheat or rice, much attention has been given to increasing the number of the grains that grow in each head, whilst causing the plant to grow with a shorter stem, thus wasting less energy on this inedible part of the plant. In the past such development was carried out in prolonged breeding programmes, but now the rate of development can be accelerated. A major area of the research is dedicated to finding ways of moving nitrogen fixing genes (NIF genes) into agricultural crops. Nitrates are vital nutrients for most plants, and some bacteria are particularly good at creating them by biochemically reducing nitrogen. The gene, which produces the necessary NIF enzymes has been isolated, sequenced and cloned in *E. coli*. Now scientists are looking for ways of placing them into cells in crops such as wheat or rice. This would enable these crops to fertilize themselves effectively, saving much money and increasing yields. Placing the gene in crops grown in developing countries would have enormous impact on their ability to grow food, as currently they cannot afford the nitrogenous fertilizers used in more affluent countries.

Resistance to disease, pest or herbicides: Many crops are particularly vulnerable to attack from specific organisms, e.g., tomatoes are attacked by aphids, potatoes by viruses, and wheat by fungi. Farmers fight back by spraying their crops with chemicals that destroy the invading organisms. Now recombinant DNA technology is allowing plant breeders to introduce genes into crops, causing them to produce their own pesticides. For examples, a tomato plant had a gene placed in it that produces a protein, which kills tomato fruit worms. When modified tomato plants and normal plants were grown together in a laboratory and deliberately infected with fruit worms and pin worms, the normal plants were stripped of their leaves, but the modified plants were unaffected. In a similar way, crops can be adapted to make them resistant to herbicides. Most herbicides operate by blocking an enzyme that is vital for the plant's survival. The idea is to equip plants with a gene, which produces an enzyme that can destroy the herbicide. When the field is spread with

the specific herbicide, the genetically modified plant will be unscathed, whilst all other plants will be killed. The first transgenic plant licensed for agricultural use in Europe (1994) was a tobacco plant that has built-in resistance to the herbicide, bromoxylnil.

Novel Crops: One of the problems of the modern agriculture is that a crop is frequently grown many hundreds or thousand of miles from the customer. It can be difficult to get it to shops before it rots. Fruit and vegetables are often harvested before they ripe and then ripened artificially just before they are sold. This leads to a loss of flavour. In the case of tomatoes, the problem may now have been solved—researchers have now produced a tomato that is resistant to becoming soft and rotting once it has ripened. Fruit normally softens because it produces an enzyme called polygalacturonase (PG). This enzyme breaks down pectin in cell walls and so the fruit becomes soft. By inserting a gene, which has a sequence that is exactly the opposite of the gene coding for PG (an antisense gene), the cells produce an antisense strand of mRNA as well as a sense strand from the normal gene. These two mRNA strands are so perfectly matched that they stick together, thus rendering the sense strand useless. Without the mRNA, no enzyme forms, and without the enzyme the fruit stays firm.

Industrial Applications

Production of Drugs: Perhaps the most glamorous aspect of recombinant DNA technology is its use to construct microorganisms, which synthesize human proteins with therapeutic potential as listed in Table 2.5. Several antibiotics can be overproduced by this technique, thereby reducing production costs. Another examples are human insulin, human growth hormone (Somatostatin) and an antiviral agent, interferon, which are being marketed. Many more are undergoing clinical trial. The interferon reduces the duration of viral infection, is effective against herpes virus infection of eye, reduces the incidence of attacks of multiple sclerosis, suppresses atherosclerosis in rats on high cholesterol diets, and is being examined for its antitumor activity. In 1985, tumor necrosis factor, a powerful anticancer substance in rats was cloned and animal studies began shortly afterwards. Interleukin-2, a substance that stimulates multiplication of certain cells in immune system, has also been cloned. It is being tested on patients with AIDS and other viral diseases and shows promise in shrinking cancers in humans.

Table 2.5 Some recombinant DNA products in medicine.

Product	*Examples/uses*
Anticoagulants	Tissue plasminogen activator (TPA); activates plasmin, an enzyme involved in dissolving clots; effective in treating heart attack patients.
Blood factors	Factor VIII; promotes clotting; it is deficient in hemophiliacs; treatment with factor VIII produced by recombinant DNA technology eliminates infection risks associated with blood transfusions.
Colorry-stimulating factors	Immune system growth factors that stimulate leukocyte production; treatmetnt of immune deficlencies and infections,
Erythropoietin	Stimulates erythrocyte production treatment of anemia in patients with kidney disease.

Contd.

Growth factors	Stimulate differentiation and growth of various cell types; promote wound healing.
Human growth homone	Treatment of dwarfism.
Human insulin	Treatment of diabetes.
Interferons	Interfere with viral reproduction; used to treat some cancers.
Interteukins	Activate and stimulate different classes of leukocytes; possible uses in treatment of wounds, HIV Infection, cancer, and immune deflciencles.
Monoclonal antibodies	Extraordinary binding specificity is used in: tumors as a cancer therapy; many other applications.
Superoxide dismutase	Prevents tissue damage from reactive oxygen species when tissues briefly deprived of O_2 during surgery suddenly have blood flow restored.
Vaccines	Proteins derived from viral coats are as effective in "priming" an immune system as is the killed virus more traditionally used for vaccines, and are safer; first developed was the vaccine for hepatitis B.

Production of synthetic vaccines: A major breakthrough in disease prevention has been the development of synthetic vaccines. Attempts have also been made to use genetic engineering to facilitate vaccine production. Production of certain vaccines such as anti-hepatitis B has been difficult because of extreme hazards of working with large quantities of the virus. The danger would be avoided if viral antigen could be cloned and purified in *E. coli* or yeast, because the pure antigen could be given as a vaccine, which has received regulatory approval from the U.S. food and drug administration. Now genes for the major subunits of a number of viruses e.g., foot and mouth disease virus, have been cloned in *E. coli*, but the results have been disappointing because peptides thus produced have been either poorly immunogenic or thermally instable. However, the use of vaccinia virus (the anti-smallpox agent) as a carrier has been fruitful. The procedure makes use of the fact that viral antigens are on surface of virus particles and some of these antigens can be engineered into the coat of vaccinia. By 1985, vaccinia hybrids (containing vaccinia DNA and viral antigen, which are produced by genetic recombination of normal vaccinia DNA and plasmid DNA containing viral-antigen gene in animal cell lines) with surface antigens of hepatitis B, influenza virus and vesicular stomatitis virus have been prepared and shown to be useful vaccines in animal tests. A surface antigen of *Plasmodium falciparum* (causative agent of malaria) has also been placed in the vaccine coat, which may lead to an antimalarial vaccine.

Food industries: We have already seen how recombinant DNA technology is affecting food production in standard agricultural situations. Now we turn to the other areas of the food industry that are making use of this technique. For example, bacteria can be designed to grow on virtually any energy-rich molecules. Some have been adapted to use methane gas as a nutrient and others grow successfully on paper pulp. This is already providing a new source of protein to the food industry. The growing bacteria can be harvested and their proteins are purified. These proteins may be of particular value as the numbers of people eating a vegetarian diet increase. This has two benefits: firstly, growing farm animals to supply us with protein is a very inefficient use of energy as it can take between 10 to 20 kg of protein in feeds to produce 1 kg of meat. However, bacteria are much more efficient. Secondly, waste materials such as pulped newspapers could form the basic nutrient supply for new bacteria. In addition, industrial cheese production uses a lot of enzyme called chymosin (commonly called rennin) to coagulate the protein casein, found in milk. Traditionally, this enzyme is obtained from the content of the abomasums (fourth stomach) of calf.

Bacteria are modified by transforming them with the inclusion of a gene that causes chymosin production for making the vegetarian cheese.

Other Biotechnological Applications

DNA sequence analysis: A great deal of information can be gained about a gene's operation once the sequence of bases is known. This was made possible because, like so many aspects of recombinant DNA technology, the process leads itself to automation. It requires homogeneous duplex segments that are produced by cloning because sequencing by Maxam-Gilbert or Sanger's methods requires microgram quantities (Figure 2.7).

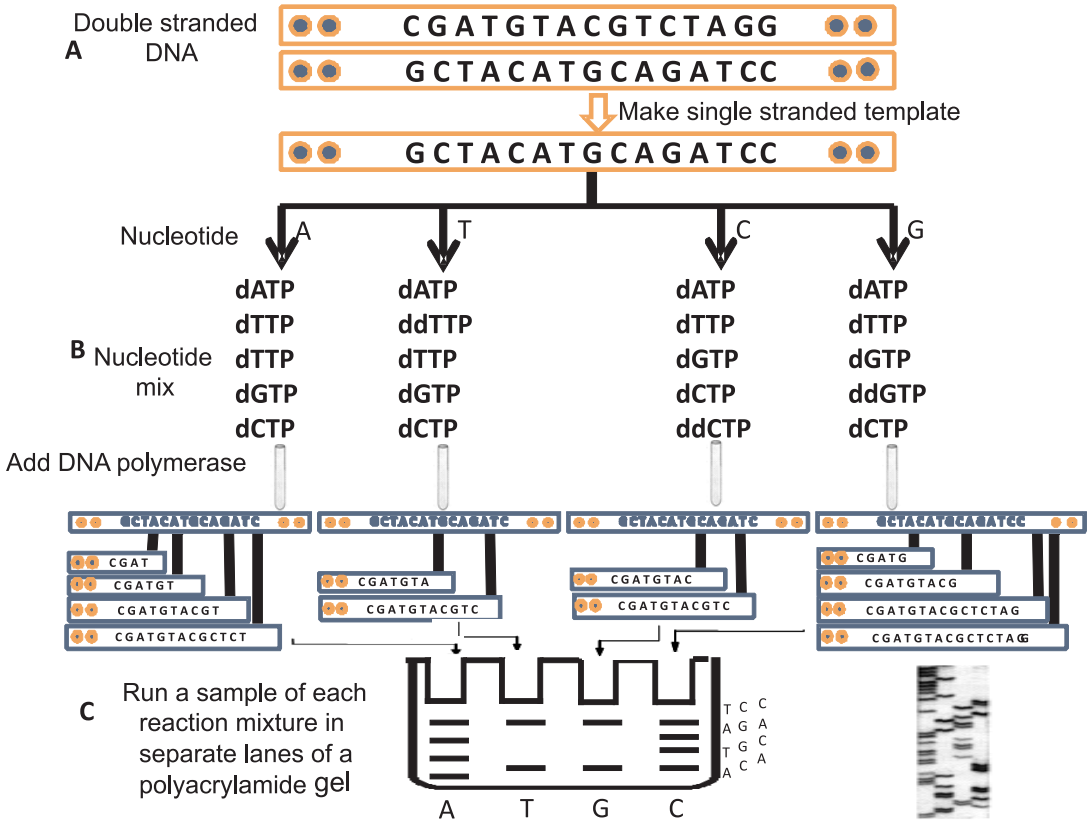


Figure 2.7 The Sanger dideoxy DNA sequencing procedure.

Site directed mutagenesis: Small regions of DNA can be specifically modified and reintroduced into the organism. This process is known as site-directed mutagenesis. After modification, the altered DNA can be amplified in bacteria and effect of the alterations can be studied either *in vitro* or *in vivo.* Specific amino acids may be replaced individually by site-directed mutagenesis. This powerful approach to study protein structure and function changes the amino acid sequence of a protein by altering the DNA sequence of the cloned gene (Figure 2.8). A short synthetic DNA strand with a specific base change is annealed to a single-stranded copy of the cloned gene within a suitable vector. The mismatch of a single base pair in 15 to 20 bp does not prevent annealing if

it is done at an appropriate temperature. The annealed strand serves as a primer for the synthesis of a strand complementary to the plasmid vector. This slightly mismatched duplex recombinant plasmid is then used to transform bacteria, where the mismatch is repaired by cellular DNA repair enzymes. About half of the repair events will remove and replace the altered base and restore the gene to its original sequence; the other half will remove and replace the normal base, retaining the desired mutation. Transformants are screened until a bacterial colony containing a plasmid with the altered sequence is found. Parts of two different genes can also be ligated to create new combinations. The product of such a fused gene is called a fusion protein. Researchers have now developed ingenious methods to bring about virtually any genetic alteration *in vitro*. Reintroduction of the altered DNA into the cell permits investigation of the consequences of the alteration. Site directed mutagenesis has greatly facilitated research on proteins by making specific changes in the

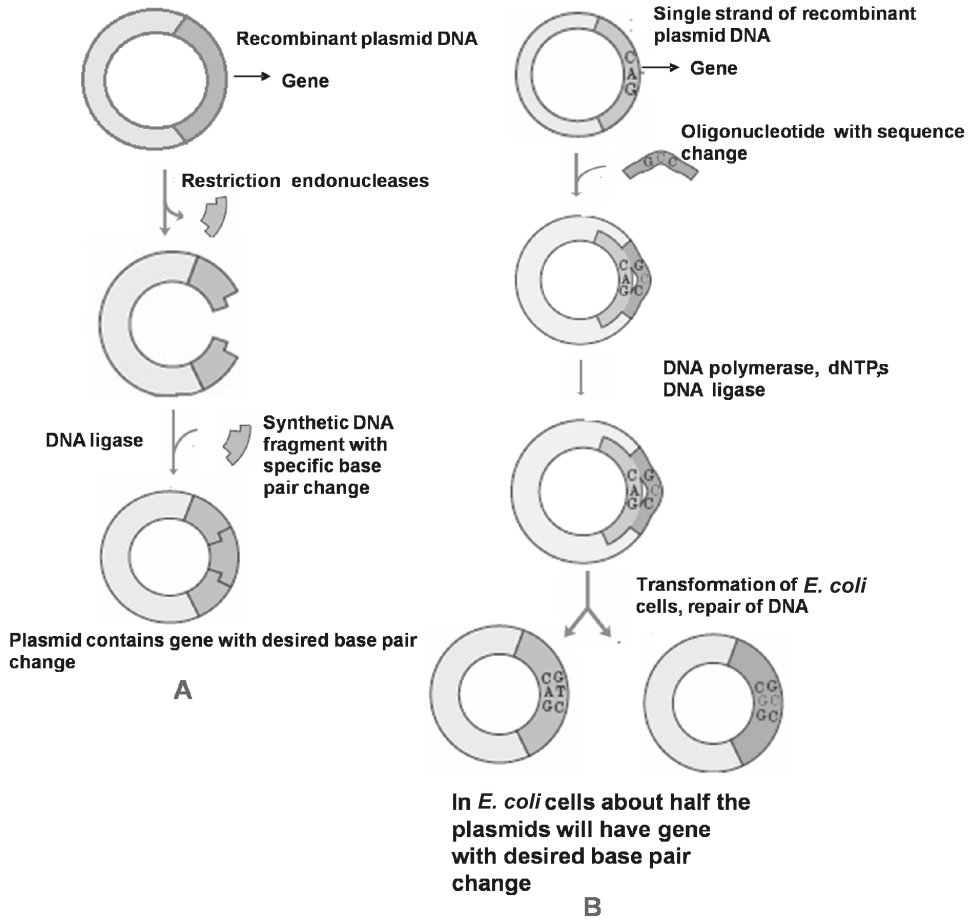


Figure 2.8 Two approaches for site-directed mutagenesis. (a) A synthetic DNA segment replaces a DNA fragment that has been removed by cleavage with a restriction endonuclease. (b) A synthetic oligonucleotide with a desired sequence change at one position is hybridized to a single-stranded copy of the gene to be altered.

primary structure of a protein and to examine the effects of these changes on the folding, three-dimensional structure, and activity of the protein.

Gene mapping: Studies using cloned DNA, e.g., *Saccharomyces cerevisiae* even in host were homologous between plasmid and host DNA not occur. One important approach involves the development of the technique known as Chromosome Walking. This is a process in which a set of cloned DNAs, is isolated so that a contiguous region of the genome is represented in the population of the clones. Chromosome walking is very useful for investigating the organization of genes in complex Eukaryotes, for which detailed genetic maps are almost impossible to obtain by other means. For example, Human Genome Organization (HUGO) project aims to produce a map of the entire genome on the 46 human chromosomes. The genome has been divided into a number of different regions, and some 250 individual laboratories around the world are working on sequencing each part. The potential benefit for medical is immense, as it will accelerate the rate at which genes can be located. Once the genes are located, genetic screening for many more conditions will be possible. In addition, it will increase our understanding of the codes that surround genes and control their activity.

Forensic science: When investigating a criminal offence, such as a burglary, serious sexual assault or murder, the police try to find evidence that will indicate who the criminals are. Recombinant DNA technology has now been used in a number of different ways. The best known example is DNA fingerprinting, which is based on two assumptions: firstly, each of a person's cells carries an identical set of DNA, and secondly, the DNA code for every individual person is unique. The sequences that code for genes are very similar throughout the population. However, sequence of DNA contain short sequences (15-100 bp) that repeat over and over again. The number of repeats in these areas of DNA are very different between individual people. Oligonucleotide probes, which code for one of the repeating sequences are used, each person's DNA producing a unique pattern. This means that if a few drops of blood or a few hair follicle cells attached to pieces of hair are left at the scene of crime, the DNA can be extracted, multiplied and then analyzed. The results can then be matched to those of a similar analysis carried out on samples taken from a suspect. If they match, this indicates that the suspect was at the crime site.

FUTURE PROSPECTS OF RECOMBINANT DNA TECHNOLOGY

The advent and development of recombinant DNA has been portrayed as very much a mixed blessing for mankind. A whole proponent have hailed it as a source of technology that will some day solve many of the problems of environmental pollution, food and energy shortages and human diseases. Gene manipulation opens the prospects of constructing bacterial cells, which can grow easily and inexpensively, and synthesize a variety of biological products like antibodies, hormones, enzymes, etc. In contrary, its opponents have bitterly criticized research in this area because of possibilities for accidental development and release of highly virulent forms of infective agents that may lead to epidemic diseases of unknown properties. Possibility exists that potentially biohazardous consequences might result from widespread injuditicious use of constructing novel organisms containing combinations of toxin producing capabilities and showing antibiotic resistance. Experiment involved in the introduction of DNA from tumor viruses into bacteria and

infection of these bacteria to human beings will increase the incidence of cancer. Newly developed cloning methods offer the prospect of dealing with a wide variety of scientific and medical problems as well as other problems that trouble society.

CONCLUSION

The importance of recombinant DNA technology can be judged by the diversity of application and high market potential. There are more than 726 companies in the USA dealing with the products emanating from the application of genetic engineering. The Human Genome Project has relied on the recombinant DNA technology to generate libraries of genomic DNA molecules. Proteins for the treatment or diagnosis of disease have been produced using recombinant DNA techniques. Till date, over eighty products that are currently used for treatment of diseases or vaccination have been produced using recombinant DNA techniques. At least 350 additional recombinant-based drugs are currently being tested for safety and efficacy. Recombinant DNA technology has also been used to produce genetically modified foods with improved nutritional qualities. It is likely that this relatively new area of genetics will continue to play an increasingly important part in biological research into the foreseeable future. The basic information emerging out of this technology involves organization of the chromosomes, genetic switches in development and differentiation leading to amplification of genes which will have profound significance.

FURTHER READINGS

1. Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B. and Moreno, R.F. (1991) Complementary DNA sequencing: expressed sequence tags and Human Genome Project. *Science.* **(252):** 1651-1656.
2. Alberts B., Johnson A., Lewis J., Raff M., Roberts K. and Walter P. (2002) *Molecular Biology of Cell*. Garland, New York (ISBN 0-8153-4072-9).
3. Alwine J.C., Kemp D. J. and Stark G.R. (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzylomethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci*. **(74):** 5463-5354.
4. Arat S., Rzucidlo S.J., Gibbons J., Miyoshi K. and Stice S.L. (2001) Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. *Mol. Reprod. Dev.* 60 **(1):** 20-26.
5. Archer J., Pinney J.W., Fan J., Simon-Loriere E., Arts E.J., Negroni M. and Robertson D.L. (2008) Identifying the Important HIV-1 Recombination Breakpoints. *PLoS Comput. Biol.* 4 **(9):** 100-117.
6. Avery O.T., MacLeod C.M. and McCarty M. (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. Exp. Med.* **(79):** 137-158.
7. Bamshad, M. and Wooding, S.P. (2003) Signatures of natural selection in the human genome. *Nat. Rev. Genet.* **(4):** 99-111.
8. Brenner, S. (2004) Genes to genomics. *Annu. Rev. Genet.* **(38):** 20-25.
9. Brummell D.A. and Harpster M.H. (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol.* **(47):** 311-340.
10. Capecchi M.R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell.* **(22):** 479-488.

11. Capper J.L., Castañeda-Gutiérrez E., Cady R.A. and Bauman D.E. (2008) The environmental impact of recombinant bovine somatotropin (rbST) use in dairy production. *Proc. Natl. Acad. Sci. USA.* 105 **(28):** 9668-9673.
12. Carbonaro D.A., Jin X., Cotoi D., Mi T., Yu X.J., Skelton D.C., Dorey F., Kellems R.E., Blackburn M.R. and Kohn D.B. (2008) Neonatal bone marrow transplantation of ADA-deficient SCID mice results in immunologic reconstitution despite low levels of engraftment and an absence of selective donor T lymphoid expansion. *Blood.* 111 **(12):** 5745-5754.
13. Cavazzana-Calvo M., Thrasher A. and Mavilio F. (2004) The future of gene therapy. *Nature.* 427 **(6977):** 779-781.
14. Clark, M.S. (1999) Comparative genomics: the key to understanding the human genome project. *Bioassays.* **(21):** 121-130.
15. Cohen S., Chang A., Boyer H. and Helgfing R. (1973) Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci.* **(70):** 3240-3244.
16. Collins, F.S., Green, E.D., Guttmacher, A.E., and Guyer, M.S (2003) A vision for the future of genomics research. *Nature.* **(422):** 835-847.
17. Colowick, S. P. and Kapian, ON (1980) *Methods in Enzymology* - Volume 68; *Recombinant DNA*. Academic Press, ISBN 012181968X.
18. Cooper, Geoffrey (1997) *The Cell: A Molecular Approach.* Washington, DC: ASM Press.
19. David L. Nelson and Michael M. Cox (2001) Lehninger Principles of Biochemistry Fourth Edition University of Wisconsin–Madison) California, San Diego.
20. Felgner, P.L. T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold and M. Danielsen (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Nat. Acad. Sci.* **(84):** 7413-7417.
21. Garret, R.H. and Grisham, C.M. (2000) *Recombinant DNA Technology is also known as Transgenic Biochemistry,* Saunders College Publishers, (ISBN 0030758173).
22. Glick, Bernard and Jack Pasternak. (1998) *Molecular Biotechnology: Principles and Applications of Recombinant DNA,* 2nd ed. Washington, DC: ASM Press.
23. Grunstein M. and Hogness D.S. (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.* **(72):** 3961-3965.
24. Gunther M., Wagner E. and Ogris M. (2005) Specific targets in tumor tissue for the delivery of therapeutic genes. *Curr. Med. Chem. Anti. Canc. Agents.* 5 **(2):** 157-171.
25. Hansen, G. and Wright M.S. (1999) Recent advances in the transformation of plants. *Trends Plant Sci.* **(4):** 226-231.
26. Heyman Y., Zhou Q., Lebourhis D., Chavatte-Palmer P., Renard J.P. and Vignon X. (2002) Novel approaches and hurdles to somatic cloning in cattle. *Cloning Stem Cells.* 4 **(1):** 47-55.
27. Hohn B. and Murray K. (1977) Packaging recombinant DNA molecules into bacteriphage particles *in vitro. Proc. Natl. Acad. Sci.* **(74):** 3259-3263.
28. Houdebine L.M. (2003) *Animal Transgenesis and Cloning.* John Wiley & Sons, Inc. Hoboken, NJ, USA, pp. 34-73.
29. Jackson, D.A., Symons, R.H and Berg, P (1972) Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **(69):** 2904-2909.
30. James D.W., Richard M.M., Amy A.C. and Jan A.W. (2007) *Recombinant DNA: Genes and Genomes–A Short Course*. Cold Spring Harbor Laboratory Press, New York.
31. Jeremy M. Berg, John L. Tymoczko and Lubert Stryer (1992) *Biochemistry.* San Francisco: W. H. Freeman., 2 Rev. edition (ISBN 0-7167-8724-5).
32. Kikkert J.R., Vidal J.R. and Reisch B.I. (2005) Stable transformation of plant cells by particle bombardment biolistics. *Methods Mol Biol.* **(286):** 61-78.

33. Klein T.M., Wolf E.D., Wu R. and Sanford J.C. (1987) Hugh-velocity microprojectiles for delivering nucleic acids into living cells. *Nature.* **(327):** 70-73.
34. Kreuzer, Helen, and Adrianne Massey (2000) *Recombinant DNA and Biotechnology,* 2nd ed. Washington, DC: ASM Press.
35. Kunkel T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. U S A.* 82 **(2):** 488-492.
36. Lai C.M., Lai Y.K. and Rakoczy P.E. (2002) Adenovirus and adeno-associated virus vectors. *DNA Cell Biol.* 21 **(12):** 895-913.
37. Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., *et al*. (2001) Initial sequencing and analysis of the human genome. *Nature.* **(409):** 860-921.
38. Lanza R.P., Dresser B.L. and Damiani P. (2002) *Cloning Noah's ark, in Understanding Cloning*. Scientific American, Inc. and Byron Press Visual Publications, Inc, 24-35.
39. Lobban P and Kaiser AD (1973) Enzymatic end-to-end joining of DNA molecules. *J. Mol. Biol.* **(78):** 453-471.
40. Lodish H., Berk A., Zipursky S.L., Matsudaira P., Baltimore D. and Darnell J. (2003) *Molecular Cell Biology.* W.H. Freeman, New York.
41. Lorence A. and Verpoorte R. (2004) Gene transfer and expression in plants. *Methods Mol. Biol.* **(267):** 329-50.
42. Lynch S.V. and Wiener-Kronish J.P. (2008) Novel strategies to combat bacterial virulence. *Curr. Opin. Crit. Care*. 14 **(5):** 593-599.
43. Ma H. (2004a) Technique of animal clone. *Nature and Science.* 2 **(1):** 29-35.
44. Mahowald, M.B., Verp, M.S. and Anderson R.R. (1998) Genetic counselling: clinical and ethical challenges. *Annu. Rev. Genet.* **(32):** 547-559.
45. Maxam A.M. and Gilbert W. (1977) A new method of sequencing DNA. *Proc. Natl. Acad. Sci.* (74) 560-564.
46. McCutchan J.H. and Pagano J.S. (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer. Inst.* 41 **(2):** 351-357.
47. Meselson M. and Yuan R. (1968) DNA restriction enzyme from *E. coli. Nature.* **(217):** 1110-114.
48. Nathan P. Kaplan, Nathan P. Colowick and Ray Wu (1980) *Recombinant DNA,* Vol. 68: *Recombinant D* Part F (*Methods in Enzymology*). Academic Press (ISBN 0-1218-1968-X).
49. Ohlstein, E.H., Ruffolo, R.R., Jr. and Elliott JD (2000) Drug discovery in the next millennium. *Annu. Rev. Pharmacol. Toxicol.* **(40):** 177-191.
50. Old, R.W., and S.B. Primrose (1994) *Principles of Gene Manipulation,* 5th ed. London: Blackwell Scientific Publications.
51. Pagano J.S. and Vaheri A. (1965) Enhancement of infectivity of poliovirus RNA with diethylaminoethyl-dextran (DEAE-D). *Arch Gesamte Virusforsch.* 17 **(3):** 456-464.
52. Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C., and Evans, R.M. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature.* **(300):** 611-615.
53. Peppin L., McEwing R., Carvalho G.R. and Ogden R.A. (2008) *DNA-Based Approach for the Forensic Identification of Asiatic Black Bear* (Ursus thibetanus) in a Traditional Asian Medicine [In press].
54. Peter Moore (1994) Recombinant DNA technology. The Biochemical Society, 59 Portland Place, London WIN 3AJ (ISBN 0-90449-8302).
55. Pfeifer, A. and Verma, I.M. (2001) Gene therapy: promises and problems. *Annu. Rev. Genomics Hum. Genet.* **(2):** 177-211.

56. Pinto G.A., Giordano R.L. and Giordano R.C. (2008) Remote engineering for a cheese whey biorefinery: an Internet-based application for process design, economic analysis, monitoring, and control of multiple plant sites. *Bioprocess Biosyst. Eng.* [In press].
57. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
58. Sanger F., Nicklen S. and Coulson A.R. (1977) A rapid method for determining sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* **(74):** 5463-5467.
59. Shimizu M., Goto M., Hanai M., Shimizu T., Izawa N., Kanamoto H., Tomizawa K., Yokota A. and Kobayashi H. (2008) Selectable tolerance to herbicides by mutated acetolactate synthase genes integrated into the chloroplast genome of tobacco. *Plant Physiol.* 147 **(4):** 1976-1983. [In press].
60. Smith C.J.S., Watson C.P., MOlTis P.C., Bird C.R., Seymour G.B., Gray J.E., Arnold C., Tucker G.A., Schuch W., Harding S. and Grierson D. (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant Mol. Biol.* **(14):** 369-379.
61. Smith H.O. and Wilox K.W. (1970) A restriction enzyme from *Hemophilus influenza*, I. Purification and general properties. *J. Mol. Biol.* **(51):** 373-391.
62. Southern E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **(98):** 503-517.
63. Tetteh K.K. and Polley S.D. (2007) Progress and challenges towards the development of malaria vaccines. *BioDrugs.* 21 **(6):** 357-373.
64. Umemoto Y., Sasaki S., Kojima Y., Kubota H., Kaneko T., Hayashi Y. and Kohri K. (2005) Gene transfer to mouse testes by electroporation and its influence on spermatogenesis. *J. Androl.* 26 **(2):** 264-271.
65. Uzogara S.G. (2000) The impact of genetic modification of human foods in the 21st century: a review. *Biotechnol Adv.* 18 **(3):** 179-206.
66. Wadhwa, P.D., Zielske, S.P., Roth, J.C., Ballas, C.B., Bowman, J.E. and Gerson, S.L. (2002) Cancer gene therapy: scientific basis. *Annu. Rev. Med.* **(53):** 437-452.
67. Wakabayashi H., Su Y.C., Ahmad S.S., Walsh P.N. and Fay P.J. (2005) A Glu113Ala mutation within a factor VIII Ca2+-binding site enhances cofactor interactions in factor Xase. *Biochemistry.* **(44):** 10298-10304.
68. Warden D. and Thorne H.V. (1968) The infectivity of polyoma virus DNA for mouse embryo cells in the presence of diethylaminoethyl-dextran. *J. Gen. Virol.* 3 **(3):** 371-377.
69. Watson J.D. and Crick F.H.C. (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature.* **(171):** 737-738.
70. Yan Y., Yang J., Dou Y., Chen M., Ping S., Peng J., Lu W., Zhang W., Yao Z., Li H., Liu W., He S., Geng L., Zhang X., Yang F., Yu H., Zhan Y., Li D., Lin Z., Wang Y., Elmerich C., Lin M. and Jin Q. (2008) Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated Pseudomonas stutzeri A1501: *Proc. Natl. Acad. Sci. U S A.* 105 **(21):** 7564-7569.
71. Zambryski, P., Tempe J. and Schell J. (1989) Transfer and function of T-DNA genes from agrobacterium Ti and Ri plasmids in plants. *Cell.* **(56):** 193-201.
72. Zhu, H., Bilgin, M. and Snyder, M. (2003) Proteomics. *Annu. Rev. Biochem.* **(72):** 783-812.

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